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TECHNICAL NOTE

A simple fluorometric method for glucose determination in nanoliter samples

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In micropuncture study of the kidney, it is necessary to analyze renal tubule fluid samples in nanoliter quantities. Although microanalytical methods for glucose in such volumes have been described [1], these require special designs of microcuvettes as well as expensive equipment that may not be readily available to many laboratories. Here, a simple fluorometric hexokinase method for glucose is described using commercially available devices and relatively inexpensive equipment.

The enzymatic glucose determination involves coupled reactions, which ultimately produce nicotinamide adenine dinucleotide, reduced form ($NADH_2$), by the following two steps:

$$\text{Glucose} + \text{ATP} \xrightarrow[Mg^{++}]{\text{hexokinase}} \text{glucose-6-phosphate} + \text{ADP}.$$

$$\text{Glucose-6-phosphate} + \text{NAD} \xrightarrow{\text{G-6-PDH}} \text{6-phosphogluconate} + NADH_2.$$

One picomole of $NADH_2$ is produced for each picomole of glucose in the original sample. The fluoresence of $NADH_2$ excited by a spectrum of light at 350 nm is measured at 450 nm.

To constitute the reagent solution, one tablet of Eskalab® (Smith Kline Instruments, Inc., Sunnyvale, Va.) was dissolved in 2 ml of distilled water that was free of microorganisms. The resulting solution had the approximate molar concentrations of the following: adenosine triphosphate (ATP), 390 μmoles/liter; nicotinamide adenine dinucleotide, oxidized form (NAD), 323 μmoles/liter; magnesium, 2 mmoles/liter; hexokinase, 525 IU/liter; glucose-6-phosphate dehydrogenase, 825 IU/liter; Tris (hydroxymethyl) aminomethane carbonate, 30 mmoles/liter; and succinic acid, 5 mmoles/liter with pH buffered at 7.5 ± 0.1. The reagent solution was kept on ice throughout the analysis.

A constant-volume transfer pipette, about 15 nl in size, was constructed according to the technique described by Prager, Bowman, and Vurek [2]. Microcuvettes, adapted for use in the fluorocolorimeter (American Instrument Co., Silver Spring, Md.), were made by cutting 100-μl constant-bore glass capillaries (Microcaps® disposable pipettes, Drummond Scientific Co., Broomall, Penn.) in half. These were cleaned by soaking them in 70% nitric acid overnight. The acid was removed by 10 to 15 rinses with distilled water. Care was taken to completely remove the water by vacuum aspiration. The microcuvettes were dried in an oven at 150° C and stored in a covered beaker.

The reagent pipette was made by heat-tapering both ends of a clean, dry glass capillary tube (Curtin-Mathison Scientific, Inc., Maryland Heights, Mo.). The length of the nontapered portion was about 1.3 cm, and the I.D. was 0.4 mm. The pipette was placed in a Microcap® holder (supplied with Microcaps) fitted with polyethylene tubing which was attached to a glass syringe. This allowed the transfer of a constant volume of reagent (approximately 1.6 μl) to the end of a microcuvette.

A modification of the "cuvette filling apparatus" described by Vurek and Pegram [3] was used. Glass capillary tubes were filled with mineral oil colored

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with Sudan Black dye, and then placed in a holder. Using a stereo-zoom microscope (Bausch and Lomb), we introduced glucose samples to oil-filled tubes from glass capillary pipettes attached to a micromanipulator and connected to a syringe. The sample was introduced such that it split the column of oil. When only a very small quantity of sample was available, the bubble of sample was placed on the inside wall of the tube. Using the constant volume transfer pipette, we then transferred about 15 nl of sample to a constant volume of reagent that had been previously delivered into a Microcap as described above. The constant volume transfer pipette, which was used for all samples and standards, was washed with chromic acid before each use, thoroughly rinsed with distilled water, and dried by forcing air through the pipette with a syringe.

The microcuvette containing reagent-sample solution at one end was then removed from the holder and the solution was allowed to drop to the midsection of the cuvette. To avoid erratic results from heating the solution, only the dry end of the cuvette was flame-sealed. The wet end was covered with a small rubber cap (Critocap®, Sherwood Medical Industries, Inc., St. Louis, Mo.), to prevent evaporation. The solution was immediately centrifuged to ward the dry end of the cuvette for further mixing, and frozen for storage until all reagent-sample solutions were prepared.

The solutions were then thawed at room temperature and read in the fluoro-colorimeter. Using a mercury vapor lamp as the light source, we set the excitation maximum at 350 nm with a pyrex filter (Corning) and the fluoresence maximum at 450 nm with an interference filter (Infrared Industries, Inc. Waltham, Mass.). In order to obtain proper resolution in fluorometry within a glucose concentration range of 1 to 5 mmoles/liter, a narrow but adequate amount of light must enter the central portion of the solution without excessive reflection from the meniscus, the sealed end, or the glass wall of the capillary cuvette. This was accomplished by modifying the instrument's microcuvette holder. The cylindrical piece inside the holder was rotated 90° to align its hole with the light source. A small piece of black plastic tape was placed over the hole and then pierced in the center by a heated 27-gauge needle. This produced a pin hole which limited the size of the light directed to the solution. The size of the pin hole was adjusted to obtain a maximum resolution in fluorometric readings for the standard solutions.

Standard solutions of glucose at concentrations 0, 1, 3, 5, and 7 mmoles/liter were prepared in distilled water. The sensitivity and linearity of a typical standard curve are demonstrated in Figure 1. The reagent-sample mixture could be frozen for weeks without changing the sensitivity.

Glucose recovery was tested in artificial tubule fluid samples that contained sodium, 140 mmoles/liter; potassium, 4 mmoles/liter; calcium, 1.5 mmoles/liter; magnesium, 0.5 mmoles/liter; chloride, 130 mmoles/liter; HPO_4, 1 mmole/liter; urea, 20 mg/100 ml; and uric acid 2 mg/100 ml, which approximate the concentrations of those in late proximal tubule fluid. Twenty-four samples ranging in glucose concentrations from 1 to 5 mmoles/liter in the artificial tubule fluid were analyzed using the micromethod. Each sample was prepared in duplicate and the average value was reported. The results are shown in Table 1. The mean recovery of glucose was 101 ± (SD) 2% with coefficient of variation at 1.96%, indicating a high degree of accuracy and reproducibility. In addition, glucose recovery by the micromethod was tested in 14 samples of dog plasma ultrafiltrate with glucose concentrations ranging from 2.9 to 7.9 mmoles/liter. The mean recovery rate was 102 ± (SD) 2%, and the coefficient of variation was 2.19%.

The glucose recovery technique was also used to measure the potential interference caused by various substances. There was no significant interference from inulin, uric acid, and ascorbic acid in high concentrations as listed in Table 2, and fructose at

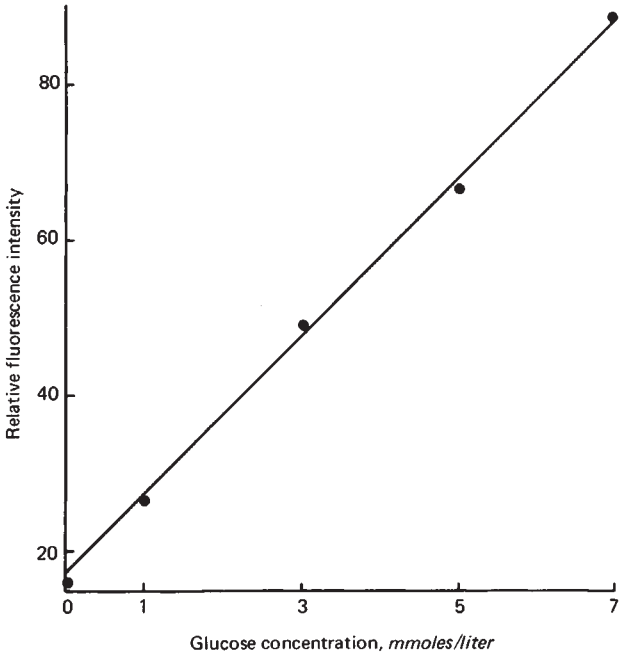

Fig. 1. *Relative fluoresence of standard glucose solutions, 15 nl in size.* Each point represents the mean of duplicate sample readings.

Table 1. Recovery of glucose in artificial tubule fluid

Sample no.	Amount added *mmoles/liter*	Amount recovered *mmoles/liter*	*%*
1	1.00	1.06	106
2	1.20	1.19	99
3	1.40	1.47	105
4	1.50	1.52	101
5	1.70	1.73	102
6	1.80	1.81	101
7	1.90	1.86	98
8	2.00	2.02	101
9	2.20	2.19	100
10	2.30	2.35	102
11	2.40	2.40	100
12	2.50	2.60	104
13	2.70	2.80	104
14	2.90	2.89	100
15	3.00	2.96	99
16	3.20	3.18	99
17	3.40	3.42	101
18	3.50	3.57	102
19	3.70	3.70	100
20	3.90	3.98	102
21	4.20	4.27	102
22	4.40	4.48	102
23	4.50	4.52	100
24	5.00	5.01	100

concentrations below 100 mg/100 ml. As seen in Table 2, fructose at concentrations above 100 mg/100 ml did alter the recovery of glucose.

For analysis of the composition of renal tubule fluid samples obtained in micropuncture study, it is necessary to use a microanalytical method that is highly sensitive in nanoliter volumes, involves relatively simple procedures, gives stable readings, and is free from interference by other substances present in the tubule fluid. The microfluorometric method for glucose using hexokinase as described above meets these criteria. The reagent consists of a mixture of a number of enzymes that are conveniently available in a tablet form. Other than the usual tediousness involved in the handling of any nanoliter-size samples, the procedure is relatively simple, including only a single step of reagent-sample mixing. The technique is highly sensitive and accurate, requiring only 15 nl of tubule fluid which normally contains glucose in concentration at about 1 mmole/liter in the late proximal tubule [4]. This indicates that the minimal glucose content detectable by the technique is approximately 10 pmoles. Adjustment of the size of the light path on the reagent-sample mixture plays a significant role in maintaining the optimal sensitivity

Table 2. Tests for interference

Test substance[a]	Concentration *mg/100 ml*	Recovery of glucose[b] *%*
Inulin (10)	1,500	102 ± 3
Fructose (10)	5,000	127 ± 54
Fructose (10)	1,000	114 ± 27
Fructose (10)	100	104 ± 5
Uric acid (10)	200	103 ± 4
Ascorbic acid (10)	50	100 ± 1

[a] The number in parentheses indicates number of samples tested.

[b] Recovery of glucose was tested at concentrations of 0, 1, 3, 5, and 7 mmoles/liter. Values are mean ± SD.

of the technique. The fluorometric technique is virtually free from interference by the agents tested except for fructose in extremely high concentrations. The latter should not constitute any problem in micropuncture experiments since fructose exists only in very low concentration in the body fluid. The fluorometer and the design of the microcuvette holder used in the present technique are all readily available commercially and relatively inexpensive. We believe that the method for glucose determination described here is simple enough to be applied to micropuncture study in most micropuncture laboratories.

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